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PRINCIPAL INVESTIGATOR: Gerald M. Kolodny, M.D.

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, MA 02215

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Gerald M. Kolodny, M.D. and Joel Yisraeli, Ph.D.

E-Mail: gkolodny@bidmc.harvard.edu and joely@ekmd.huji.ac.il

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14. ABSTRACT

We have proposed a novel technique to image and treat ovarian cancer with very high specificity, low background for imaging and low toxicity for therapy. We will make a riboswitch consisting of two aptamers and a sensor region that can hybridize with the specific intracellular ovarian cancer cell marker, VICKZ. The target for the first aptamer, EpCAM, is a surface antigen that is up-regulated in cancer cells. During cellular recycling, EpCAM will carry the attached riboswitch into the cell. Inside the cell, the riboswitch will interact with VICKZ mRNA. This interaction will change the conformation of the riboswitch to expose a second aptamer in the correct conformation to bind an administered radioactive agent which rapidly enters and exits the ovarian cancer cells, eg. antipyrine. Depending on the radioactive isotope attached to the agent, the ovarian cancer cells will be either imaged (^{123}I) or killed (^{131}I). We have successfully made both DNA and RNA aptamers to antipyrine, the 2nd proposed aptamer, and have been working on isolating aptamers to iodoantipyrine. We have also accomplished the important task of introducing a large portion of the eventual riboswitch into the cell interior where it appears to binds selectively to the target cancer cell marker, VICKZ mRNA.

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INTRODUCTION

We have proposed a novel technique to image and treat ovarian cancer with very high specificity, low background for imaging and low toxicity for therapy. We proposed to make a riboswitch consisting of two aptamers and a sensor region that can hybridize with the specific intracellular ovarian cancer cell marker,VICKZ. The target for the first aptamer, EpCAM, is a surface antigen that is up-regulated in cancer cells. During cellular recycling, EpCAM should carry the attached riboswitch into the cell. Inside the cell, the riboswitch will interact with VICKZ mRNA. This interaction will change the conformation of the riboswitch to expose a second aptamer in the correct conformation to bind an administered radioactive agent which rapidly enters and exits the ovarian cancer cells, eg. antipyrine Depending on the radioactive isotope attached to the agent the ovarian cancer cells will be either imaged (^{123}I) or killed (^{131}I). We have successfully made both DNA and RNA aptamers to antipyrine the 2nd proposed aptamer, and have been working on isolating aptamers to iodoantipyrine. We have also accomplished the important task of introducing a large portion of the eventual riboswitch into the cell interior where it appears to binds selectively to the target cancer cell marker, VICKZ mRNA.

BODY

Tasks 1-3 – Optimization and calibration of an aptamer that binds a radioactive target

Our original research strategy describes the use of a thyroid hormone aptamer as the aptamer exposed by action of the riboswitch. This aptamer was initially selected because thyroid hormone is readily taken up by most mammalian cells, including BAT cells. It can also be readily labeled with different isotopes of iodine for imaging and quantitating UCP1. In addition, an aptamer to thyroid hormone, with a description of its isolation, had already been published prior to the time of our proposal. We worked for several months using the RNA oligonucleotide described as an aptamer to thyroid hormone that contained modified nucleotides to provide RNAse resistance, but we were unable to show specific binding to either T3 or T4 thyroid hormone. However, we did show uptake of a fluorescently labeled construct containing an aptamer to EpCAM another cell surface marker joined to the reported thyroid aptamer.

Since the reported thyroid hormone aptamer was not functioning in our hands, we decided to build our riboswitch with an aptamer to an alternate target. We turned to antipyrine as a good candidate for the second aptamer in the riboswitch, which would be exposed upon riboswitch binding to VICKZ mRNA. Antipyrine can be readily labeled with isotopes of iodine, rapidly diffuses in and out of cells, and has a short biologic half-life. Since there are no published reports of an antipyrine aptamer we have been forced to isolate our own antipyrine aptamer. Because of these features of antipyrine, the development of an antipyrine aptamer appears to have a wide potential in the identification of any intracellular cell marker, including cancer cell markers, using the novel riboswitch methods we have proposed.

Isolating an RNA aptamer that recognizes antipyrine

The Yisraeli lab began the search for an RNA aptamer to antipyrine, using modified RNA nucleotides (2'-fluoro-pyrimidines) to inhibit RNAses. A template library was synthesized consisting of fixed, adaptor sequences flanking a 33 nucleotide long sequence constructed by random incorporation of nucleotides at each position. As shown schematically below (fig. 1), in

vitro transcription of these templates in the presence of the fluoro-modified nucleotides generated a highly complex (4^{33}~10^{20}) population of modified RNAs. After subtracting out the RNAs that bind to control beads, the remaining RNAs were bound to beads linked to aminoantipyrine via carboxyl groups, washed at low salt concentrations, and then eluted either with soluble antipyrine or high salt. Recovered RNAs were reverse transcribed and the cDNAs used as new templates for transcribing RNA for the next round of binding. Three rounds of selection were performed, and samples were sent for deep sequencing from the first and third rounds of selection (both antipyrine-eluted and high salt-eluted) and from the original, non-selected library. Initial analysis of the reads obtained from the MiSeq sequencer show that while the orginal, non-selected library is highly random, with no read appearing more than 4 times (out of approximately 5 million reads), one round of selection yields a number of reads that are enriched 100 times or more. Some of these reads contain motifs that are also significantly enriched in the RNAs after the 3rd round of selection. These sequences are now being synthesized for further analysis using a Biacore Surface Plasmon Resonance (SPR) machine. In addition, the Yisraeli lab has also bound aminoantipyrine to a Biacore chip with a carboxylated surface and made use of SPR to preparatively select for those RNAs binding to the immobilized aminoantipyrine. High quality reads were analyzed for motif enrichment using a K-mer analysis followed by clustering of the related sequences. Although both the initial, random library (used as a negative control) and the third round SELEX eluted by NaCl showed no enrichment of motifs, the first and third round SELEX eluted by AP contained motifs that were enriched up to several hundred fold. Using cluster analysis and comparing enriched reads as well, we have identified a list of 11 top candidates that are ready to be validated.

Figure 1.

Schematic representation of _high throughput sequencing to identify aptamers to antipyrine

Isolating an RNA aptamer using Cell-SELEX that recognizes cleaved MUC1 on the surface of cells

We obtained, from Prof. Danny Wreschner of the Tel Aviv University, NIH 3T3 cells transfected with a recombinant MUC1 construct that represents the cleaved MUC1 protein found to be highly enriched on the surface of carcinoma cells. Cell SELEX was performed using RNA transcribed from the random library described above. First, the parental NIH 3T3 cells were incubated with the RNA, and the RNA that was not bound to the cells was then incubated with the cells expressing the MUC1 construct. After half an hour of incubation, the cells were lyzed, treated with RNAse, and then the RNAse-resistant RNA was reverse-transcribed, amplified, and used again as a template for the next round of Cell-SELEX. To test for enrichment, at the third round, the RNA was bound to either parental cells or MUC1-expressing cells that were prevented from endocytosising the RNA by Na Azide. A fluorescent probe that recognizes the fixed sequences in the library was hybridized to the bound RNA and then the cells were analyzed by FACS. The cells expressing the MUC1 construct showed 2-fold higher fluorescence, indicating that the Cell-SELEX procedure enriched for aptamers recognizing MUC1. These RNAs have now been deep sequenced and are in the process of being analyzed.

Meanwhile, to avoid the use of modified nucleotides, the Kolodny lab began a search for DNA rather than RNA aptamers to antipyrine. A modified SELEX approach was used, followed by next generation sequencing. Using limited SELEX rounds on magnetic beads, followed by multiple parallel next generation sequencing we have successfully selected ssDNA aptamers showing highly selective binding of the aptamers to antipyrine. (manuscript in preparation).

Antipyrine is a small molecule (MW 188) which provides particular challenges for aptamer selection. We need an agent that rapidly enters and leaves normal cells and is only retained in cells with an exposed aptamer to the agent. Any other agent we would choose that rapidly enters and leaves cells would also have to be small. Since antipyrine in the doses we will be using is known to be nontoxic and readily labeled with radioactive iodine we have chosen to proceed with selection of an aptamer to this small molecule in spite of the known difficulty of selecting aptamers for small molecules.

The major difficulty with selecting aptamers for small molecules arises from the fact that aptamers are usually selected by binding one portion of the target, such as a protein, to a solid support such as magnetic beads. Another portion of the molecule far renmoved from the solid support binding site is then free to bind to aptamers during the SELEX selection process. With a small molecule such as antipyrine the binding of the molecule to the solid support often changes the conformation of the target or makes the molecule inaccessible to the potential aptamers. After considerable work with changes in binding sites, magnetic beads and linkers we were able to select aptamers to antipyrine by using aminoantipyrine conjugated with a carboxyl terminated linker on magnetic beads.

Aminoantipyrine was linked to carboxyl groups on side arms of superparamagnetic beads. After incubation of the antipyrine linked beads with a library of approximately 10^{14} 40 base random DNA sequences, bracketed with distinct, fixed 5' and 3' primers, the beads were extensively washed. The attached oligonucleotides were then eluted, amplified by PCR for only 15 rounds to reduce selection of only those oligonucleotides showing preferential PCR amplification, and used in a subsequent round of aptamer selection. After 5 rounds of SELEX selection, the resulting sequences had adapters and index sequences added for sequencing using an Illumina next generation massively parallel sequencer at the Hebrew University in Jerusalem, Israel, the home institution of the Yisraeli lab.

Data from the sequencer was analyzed using the Genome Tools tallymer software for counting, indexing and searching k-mers, obtained on the internet from Stefan Kurtz at the Center for Bioinformatics at the University of Hamburg in Hamburg, Germany. From the Illumina

sequencer we analyzed separately sequences of lengths 30, 25, 20,17,16,15,14,13,12 and 11 nucleotides. For each k-mer we searched for sequences appearing greater than 100 times. K-mers containing adaptor or primer sequence, or containing long runs of a single nucleotide were eliminated. We obtained from Integrated DNA Technologies (IDT, Coralville, Iowa) oligonucleotides corresponding to the ten most frequently appearing sequences for each k-mer (all of which were 15 nucleotides or smaller in length). The oligonucleotides were labeled with ^{32}P at the 5' end and then tested in a binding assay with aminoantipyrine joined to magnetic beads. Figure 2 shows the results of a binding assay with 12mer2, which showed a particularly low binding constant as analyzed by GraphPad Prism version 6.0 for Windows, GraphPad Software, La Jolla, California USA, www.graphpad.com.
The dissociation constant using one site saturation averaged over 3 experiments was calculated as 3.9+/-0.8 nM.

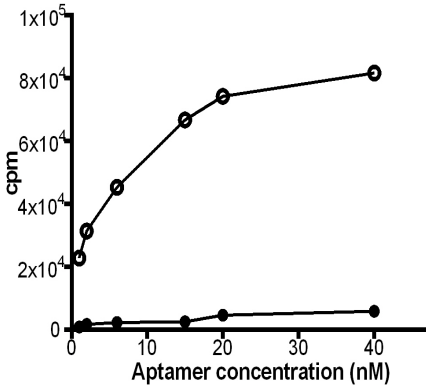

Fig. 2 Binding kinetics of antipyrine and aptamer Radiolabeled aptamer at increasing concentrations was applied to antipyrine linked to magnetic beads. The beads were washed and counted. The calculated dissociation constant was 3.9+/-0.8 nM. Open circles are cpm averaged over 3 experiments for aptamer binding to antipyrine linked to magnetic beads. Closed circles are cpm averaged over three experiments for binding to magnetic beads alone

To confirm that the sequences we had chosen were suitable aptamers, we next performed competition assays with antipyrine, aminoantipyrine and iodoantipyrine. In particular we wanted to be sure that we had selected at least one or more aptamers that would bind iodoantipyrine, since we intended to eventually use antipyrine labeled with radioactive iodine isotopes as a target for our aptamer. The targets were combined separately to each aptamer candidate and then incubated with the bead bound antipyrine. Assays with antipyrine and aminoantipyrine showed good competitive binding. However the iodoantipyrine competition assays with each of 20 candidate aptamers showed limited competition with the antipyrine bound to the beads. Because control experiments using the carboxylated beads without attached antipyrine showed very low binding of radiolabeled aptamers to iodoantipyrine, it would appear that the iodine moiety on the iodoantipyrine is interfering with the binding of the candidate aptamers.

We are currently performing deep sequencing of aptamers selected using elution of bound aptamers with iodoantipyrine. The aminoantipyrine bound to the carboxylated magnetic beads is exposed to our random DNA library. The beads are washed extensively and then incubated with iodoantipyrine to remove any aptamers that also bind to iodoantipyrine. The eluted aptamers are then amplified with PCR and reapplied to the antipyrine linked to magnetic beads for a 2nd round of SELEX selection. After six rounds of selection the aptamers are now undergoing deep sequencing

4 – Testing the ability of a surface aptamer to direct delivery of attached molecules into cells

As proof of concept that an aptamer can mediate cell uptake into cultured cells , ES2 ovarian cancer cells were exposed to a DNA EpCAM aptamer joined to Cy3-labeled DNA. Figure 3, using confocal microscopy, demonstrates increased cytoplasmic signal in cells exposed to the construct (compared to background fluorescence in control cells), indicating that the EpCAM aptamer and associated sequences is specifically endocytosed by these cells. The

presence of the fluorescence label only in the cytoplasm, and not in the nucleus, suggests that this construct has not been degraded.

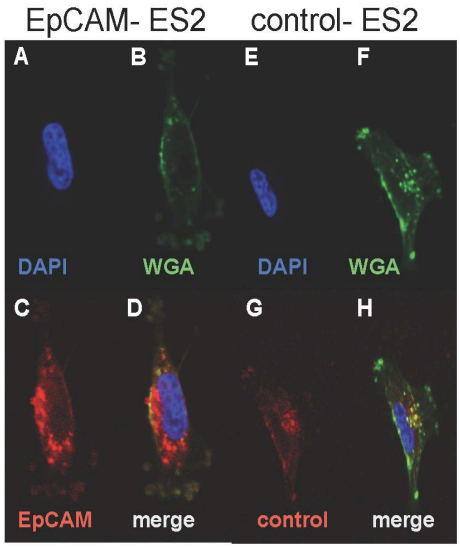

Fig. 3. Uptake of EpCAM/antiVICKZ aptamer. Fluorescently labeled antiVICKZ DNA joined to EpCAM aptamer was incubated with ES2 cells. Uptake was assayed by confocal microscopy. A-D, ES2 cells incubated with 1.8uM EpCAM-antiVICKZ labeled with texas red fluorophore. E-H, ES2 cells with no construct added. DAPI (4',6-diamidino-2-phenylindole) stained nuclei are seen in blue, WGA (wheat germ agglutinin) stained cell membranes are seen in green. Most of the ES2 cells incubated with the antiVICKZ DNA/EpCAM aptamer show increased red cytoplasmic staining compared to control cells (that show only background fluorescence), indicating specific uptake of the EpCAM-containing construct.

Fluorescence activated cell sorting (FACS) analysis, (fig. 4) also demonstrated that the EpCAM aptamer and associated sequences is efficiently endocytosed by essentially all of the cells.

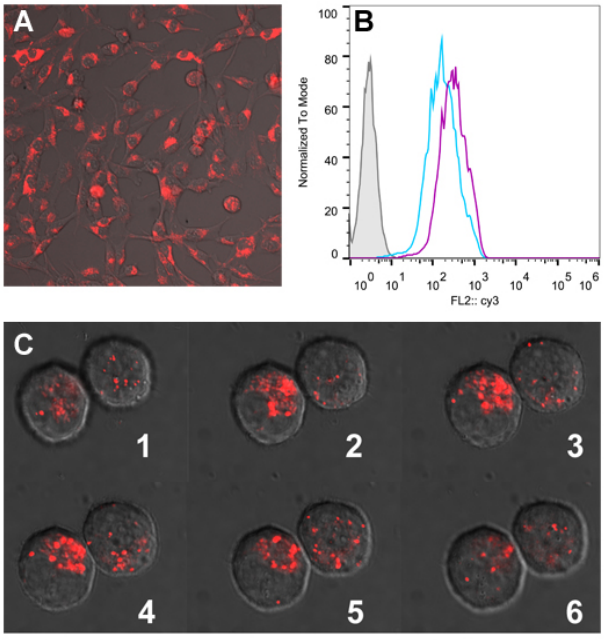

Fig. 4 Uptake of EpCAM aptamer. Fluorescently labeled DNA oligonucleotide linked to EpCAM aptamer was incubated with ES2 cells. A. ES2 cells were incubated with 200nM EpCAM-oligonucleotide labeled with Cy3. Uptake was observed by confocal microscopy (40X). B. FACS analysis was used to quantify uptake of the Cy3-EpCAM-oligonucleotide. ES2 cells were incubated overnight with no (gray), 50 nM (blue), or 200 nM (purple) Cy3-EpCAM-oligonucleotide, trypsinized, and analyzed for forward scatter and fluorescence intensity. Essentially all of the cells showed uptake of the oligonucleotide at both concentrations. C. Z-stack analysis (1-6, sections spaced 2 microns apart) of ES2 cells incubated with 200 nM Cy3-EpCAM-oligonucleotide clearly shows the presence of the label within the cell and not on the cell membrane (300X).

Task 4 – Testing the ability of an EpCAM aptamer/antiVICKZ oligo to be internalized and recognize VICKZ mRNA

The VICKZ family of proteins are associated with cell migration and movement, are abundant in many embryonic and cancer cells and help mediate ES2 migration in vitro . Having shown that the EpCAM aptamer can direct uptake into ES2 cells, we next wanted to assess the ability of the antiVICKZ sequence in this oligonucleotide to recognize and bind endogenous VICKZ RNA. We reasoned that if the DNA antiVICKZ sequence would hybridize to endogenous VICKZ RNA, the hybridized RNA would be degraded by endogenous RNAse H, leading to a VICKZ knockdown and inhibition of migration. ES2 ovarian cancer cells in culture were exposed to the EpCAM/antiVICKZ3 construct. The cells were then transferred in serum free media into Boyden chambers (tissue culture inserts with polyethylene terephthalate membrane of pore size 0.8um) in wells of serum-containing media. The established chemoattractive gradient caused the cells to migrate through the membrane toward the serum-containing media. On inspection and cell counting of up to 10 microscopic fields for each assay, the treated cells showed significant inhibition of migration compared to control untreated cells. On repeated assays the number of treated cells that migrated through the membranes was 30-50% lower than the number of untreated cells that migrated. In figure 5, control cells showed 667 cells migrating through the membrane in 10 high power microscopic fields, while the construct treated cells showed only 336 cells in ten microscopic fields. As a control for nonspecific effects of DNA either on the exterior or interior of the ES2 cells, we incubated the ES2 cells with a construct consisting of the EpCAM aptamer joined to a nonspecific DNA sequence. There was no statistically significant difference between the control cell migration and the migration of cells incubated with this construct. These results indicate that the EpCAM aptamer –anti VICKZ oligonucleotide inhibits cell migration across a membrane specifically due to the anti VICKZ sequences. We suggest that this argues for the ability of the antiVICKZ sequences to recognize and bind endogenous VICKZ RNA. Western blot analysis of extracts from the treated ES2 cells shows a 50% reduction in VICKZ3 protein expression compared to extracts from untreated control cells, using a pan-VICKZ antibody (data not shown). We are currently testing the specificity of this knockdown using antibodies that specifically recognize VICKZ1, 2, or 3, and chimeras of EpCAM aptamers with anti-VICKZ1, 2, and 3 antisense sequences.

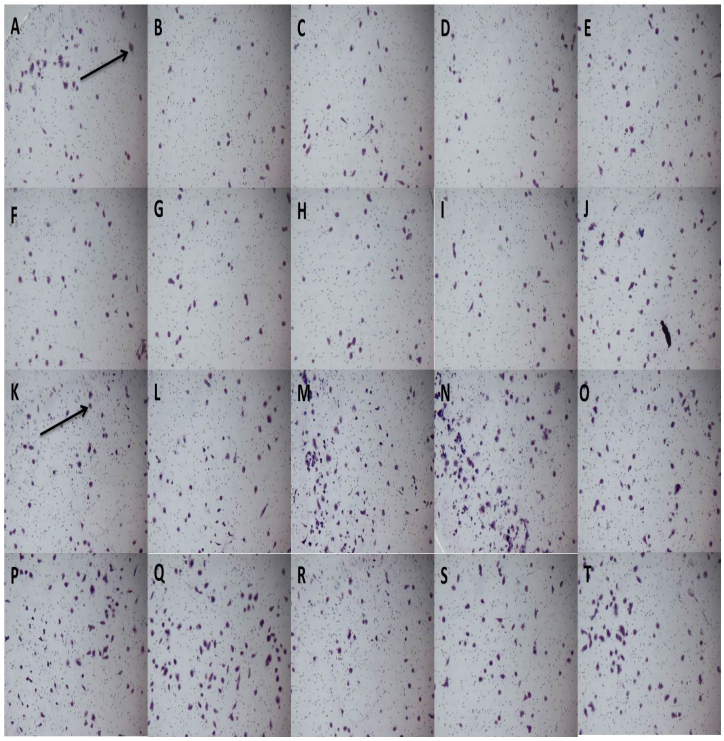

Figure 5. Migration of ES2 ovarian cancer cells. ES2 ovarian cancer cells in culture were allowed to adhere overnight to tissue culture plates and were then exposed to 1.6 uM EpCAMaptamer/antiVICKZ (panes A-J) or no construct (panes K-T) for 2 hours. The cells were trypsinized, neutralized with serum containing media, centrifuged, resuspended in serum-free media and then placed onto Boyden chambers in wells of serum-containing media for 5 hours. Non-invading cells were removed and cells were fixed and stained with Toluidine Blue. Each condition was examined under 100X magnification and cells from ten random fields were counted. Arrow in A points to cell. Arrow in K points to membrane pore. The microscopist performing the cell counting was blinded to the specific treatments. Ten ROI's were selected for each sample that encompased the entire field.

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Besides confirming that at least a portion of the eventual riboswitch could be taken up by the ES2 cells in culture, these results suggested to us the possibility of using the EpCAM/antiVICKZ DNA construct to limit cell (eg. cancer cell) mobility and metastasis of treated cells. The VICKZ family of proteins includes 3 major families of proteins. Our experiments thus far have focused on only VICKZ 3. We are now exploring the use of EpCAM aptamer joined to antiVICKZ sequences for VICKZ 1 and VICKZ 2 and a combination of all three constructs together in ES2 migration assays. If these experiments demonstrate decreased cell migration greater than 90% we will test inhibition of invasion through matrigel in the Boyden chamber and perform further control experiments before embarking on animal experiments.

KEY RESEARCH ACCOMPLISHMENTS

* **Identification of highly specific cell surface markers for brown fat cells**
- Selection of candidate antipyrine modified RNA aptamers
- Selection of candidate antipyrine DNA aptamers
- Determination of binding constants of DNA aptamers
- Demonstration of the intracellular uptake of constructs of EpCAM and portions of the riboswitch
- Demonstration of the EpCAM aptamer/antiVICKZ oligo portion of the riboswitch to recognize and hybridize with VICKZ mRNA
- Dramatic reduction in cell migration with EpCAM aptamer/antiVICKZ treatment with cells in culture.

REPORTABLE OUTCOMES

.A grant application has been submitted to the DOD CDMRP program in ovarian cancer for continued funding on this highly innovative approach to imaging and therapy of ovarian cancer. In addition grant applications for NIH funding to explore the use of riboswitch technology as we have proposed to image and treat cancer cells using radiolabeled antipyrine have been submitted. Two manuscripts are now in preparation to describe our work on the selection of antipyrine aptamers and the effects of the EpCAM aptamer/antiVICKZ DNA on cell motility.

CONCLUSION

We have selected both DNA and RNA aptamers to antipyrine and derivatives of antipyrine. We have also succeeded in showing cell internalization and functional ability of a large portion of the proposed riboswitch. After design of the folding of the riboswitch, we will be in a position to test the ability of the riboswitch to selectively bind radiolabeled targets composed of antipyrine or antipyrine derivatives. Finally this will permit us to test the ability of the cell internalized riboswitch to bind the radiolabeled targets for imaging and cell killing of ES2 ovarian cancer cells with low background uptake and low toxicity to normal cells..

As an unanticipated byproduct of our work we have found that a DNA construct consisting of an EpCAM aptamer joined to an oligonucleotide complementary to VICKZ mRNA was able to dramatically inhibit motility of ovarian cancer cells compared to untreated cells. Apparently the EpCAM aptamer portion of the construct attaches to its target on the cell surface. When the EpCAM is normally recycled into the cell interior it also internalizes the attached construct. The antiVICKZ portion of the construct then hybridizes to the VICKZ mRNA, making a DNA:RNA hybrid. The RNA portion of the hybrid is then destroyed by the normal intracellular RNAse H, resulting in decreased cell motility because of the resulting diminished VICKZ protein required

for cell motility. We intend to exploit this novel finding to determine whether this effect can be exploited to restrain ovarian cancer cell metastases in animal models.

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APPENDIX

Statement of Work

Task 1. **Optimizing the T3 aptamer.**

A number of aptamers that bind T4 have been isolated by the SELEX process. In order to identify the aptamer most likely to be effective in the context of the CO constructs, we will test the ability of these aptamers to bind T3, first in vitro and then in OC cells. These experiments will be performed by the group in Jerusalem.

1a. Double aptamer (DA) constructs will be built, consisting of an EpCam aptamer fused to a T3/T4 aptamer. ^{32}P-labeled DA constructs, will be synthesized with a biotin moiety at the 5' end, and the DAs will then be tested for their ability to bind T3-sepharose columns in vitro. These experiments should identify the shortest, effective T3 aptamer that can effectively bind T3 in vitro. (timeframe: months 1-3)

Task 2. Calibrating I^{125}-T3 uptake into, and release from, OC cells.

In parallel to identifying the optimal T3 aptamer, the Boston group will determine the kinetics of I^{125}-T3 uptake into ES2 cells. We will examine the kinetics of T3 cell uptake and discharge, and the binding to normal cell nuclear receptors, since these kinetics will determine the background to be expected after addition of CO and radiolabeled T3.

2a. ^{125}I T3 will be added to ES2 cells. At various times, cell activity will be determined. (timeframe, months 1-2)

2b. Once we have determined the time to reach steady state levels, the time it takes for the I^{125}-T3 to be released from the cells will be followed. Cells will be incubated with ^{125}I-T3 for the time determined to reach a steady state level and then they will counted at various times to determine the washout rate of the T3.(timeframe, months 3-4)

2c.. One of the sources of the background level of T3, is the binding of T3 to normal cell nuclear receptors. In our experiments it may be necessary to block these receptors by addition of blockers such as reverse T3 or tetraiodothyroacetic acid. These blockers will be added at various concentrations to ES2 cells for various times, followed by incubation with ^{125}I T3 to determine the effect of blocking the normal T3 receptors. (timeframe, months 5-6)

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Task 3. Optimizing retention within cells of I^{125}-T3 by the EpCam/T3 DA. (timeframe, months 7-13)

3a. The Boston group will determine the kinetics of ES2 cellular uptake of the EpCAM/T3 DA in comparison with the non EpCAM containing A2780 cells. ^{32}P labeled DA will be introduced into the cell media and cells solubilized at various times to determine the time to steady state level of the DA. (timeframe, months 7-8)

3b. To determine the effectiveness of theEpCAM/T3 DA constructs in cells, the constructs, with an attached biotin group, will be added to the medium of ES2 cells and allowed to be taken up by the cells for the time it was shown to result in a steady state. ^{125}I-T3 will be added to the cells, and after the time required to reach 5% of steady state levels, the cells will be washed, lyzed, and incubated with strepavidin beads that will pull down the biotinylated DAs, and any associated T3. The ability to pull down ^{125}I-T3 will be compared to DAs incubated with EpCam-negative cells and DAs fused to a shuffled T3 aptamer sequence, which is incapable of binding T3. (timeframe, months 9-13)

Task 4. **Testing an EpCam-Kras-T3 CO.**

4a The Israeli group will synthesize the EpCAM-Kras-T3 CO using the published sequences that have been shown to work in cells. The molecule will be tested in vitro, comparing synthetically synthesized wild type and mutant Kras mRNAs for their ability to activate T3 binding. (timeframe, months 4-12)

4b. The EpCAM-Kras-T3 CO will then be tested by the U.S. group in OC cells that express EpCam and mutant Kras (MDAH2774). Cells will be incubated with the CO and then incubated with ^{125}I-T3, or
the two agents incubated simultaneously and the kinetics of uptake of the T3 determined. (timeframe, months 14-19)

Task 5. Creating an EpCam-VICKZ-T3 CO.

The Israeli group will generate this CO in collaboration with our consultant in New York.

5a. We will first test the ability of a series of riboswitches recognizing VICKZ3 mRNA to function as beacons in OC cells. The beacons will be designed and tested by the New York consultant. The sequence most effective as a beacon in vivo will be used to synthesize the CO, along the lines of the EpCam-Kras-T3 CO described above. The Israeli group will test and calibrate the CO in vitro. (timeframe, months 13-24)

5b.The U.S. group will then test the EpCAM-VICKZ-T3 CO in ES2 cells, using ^{125}I –T3. Cells will be incubated with this CO and either simultaneously or later incubated with the radiolabeled T3 and then the radioactivity bound to the CO determined. The whole cells will be dissolved and counted. Separately cells incubated with biotin labeled CO will be lysed, incubated with strepdavidin beads and counted to determine activity bound to the CO only. (timeframe, months 20-24).